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Efficiency of immobilized cyanobacteria in heavy metals removal from industrial effluents

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ABSTRACT

Anabaena variabilis and *Tolypthrix ceytonica* were selected for bioremediation of heavy metals-contaminated industrial effluents based on their high efficiency as metals accumulators. Sodium alginate immobilized individual or mixed cell cultures were examined to decontaminate Plastic and electrical industrial effluents in continuous mode (at 50 and 100 ml/h flow rates) for 6 h. Removal efficiency was a function of heavy metal type, concentration, microbial species and proportionally increased with exposure time. The maximum achieved removals recorded 94.45% for Fe^{2+} (*A. variabilis* at 50 ml/h), Zn^{2+} (98.98% by *A. variabilis*; 98.63% by *A. variabilis* and *T. ceytonica* mixture and 98.61% by *T. ceytonica* at 100 ml/h), 94.22% for Pb^{2+} (*A. variabilis* and *T. ceytonica* mixture at 100 ml/h) as well as 93.33% and 91.33% for Cu^{2+} by *A. variabilis* and *T. ceytonica* at 50 and 100 ml/h flow rates, respectively. Excellent selective metals bioremoval abilities were detected by the selected cyanobacterial species. Their manipulation in the proposed biotechnology provides an economic and excellent tool for remediation of industrial effluents and protection of the received environments. Moreover, treated wastewater may be reused in any purpose such as aqua-culturing or irrigation of agricultural edible and non-edible crops.

Keywords: *Anabaena variabilis*; Bioaccumulation; Cyanobacteria; Heavy metals; Immobilization; Industrial effluents; *Tolypthrix ceytonica*

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1. Introduction

The wide distribution of heavy metals in the environment is attributed mainly to their multiple industrial, domestic, agricultural, medical, and technological applications. Metals toxicity depends on several factors including the dose, route of exposure, and chemical species, as well as the age, gender, genetics, and nutritional status of the exposed individuals. They are systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure. According to the U.S. Environmental Protection Agency, and the International Agency for Research on Cancer, they are classified as human carcinogens (known or probable) [1]. Therefore, removal of heavy metals from contaminated media considered critical necessity.

Heavy metal contamination in aquatic media can be remediated using a variety of chemical, physical, and biological technologies [2]. Biological treatment systems has attracted the attention worldwide and has helped in developing relatively efficient, low-cost, and environmentally safe treatment technologies compared with physical and chemical systems [3]. Bioremediation and biodegradation are the most promising approach for decontaminating polluted environments through exploring and engineering microbial catabolic capabilities [4] which, offer new innovative ways to clean up organic and inorganic contaminated aqueous media [5] and have the potential to eliminate or minimize health risks imposed by these hazardous waste problems [6,7].

Microalgae cultivation is a difficult task accompanied by expensive growth medium that largely limit algal industry.

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One of the recent trends is to search for new photosynthetic organisms in different environments with high growth rates, high biomass yields and high utilization potential, which could be mass cultured in wastewater reducing dependency on commercial medium while it remediates the wastewater [8]. Cyanobacteria (blue greens) ideally suited to perform these functions by virtue of their high flexibility to adapt to varies environments. Attempts toward this goal using some freshwater and marine blue-greens have been made. These features encouraged by the luxuriant growth of algal forms in eutrophic waters are a common phenomenon that can be manipulated for the removal of various kinds of inorganic and related substances through their metabolic activities [9].

Algae often serve as excellent indicators of pollution as they respond typically to many toxicants. Algae-bacterial symbiosis has long been proved to be an inexpensive process for the reclamation of wastewater [3]. The use of algae in bioremediation depends on their ability to survive upon exposure to potentially toxic treatments. Concerning metals, algae have been used as indicator organisms to identify areas of trace metal contamination because of their ability for metals absorption from one side and toxicity of some heavy metals on algae from the other side [10]. Among algae, cyanobacteria are very promising microorganisms in heavy metal uptake as well as their ability to perform oxygenic photosynthesis. Some of them have the ability to degrade many organic pollutants in one hand, and fix N_2 aerobically on the other hand. Cyanobacteria have several advantages which make them attractive hosts for biodegradative genes to enhance their biodegradation ability [5].

There are two general mechanisms of accumulating metal ions from water using aquatic plant biomass either micro or macro organisms. The first is biosorption, an energy independent binding of metals to cell wall while the second is a bioaccumulation, an energy dependent process of metal uptake into the cells [11,12]. Using living aquatic plants to sorb metals, making them less available and less dangerous, is more advantageous compared with physical and chemical methods [11,13,14]. The efficiency of bioremediation depends on the selection of microorganism [7]. For example, in 10 successive cycles, living *Chlorella miniata* achieved around 85% removal rate of Ni (initial concentration = 30 mg) in the first five cycles while *C. vulgaris* achieved around 50% only [15]. In another study, *C. pyrenoidosn* has achieved 70%–98% adsorption ability for Fe, Cu, Zn, Cd, and Pb (initial concentration = 5 mg) [16] with higher efficiency in removing single metals compared with multiple metals [17].

Physical and/or chemical pre-treatment of algal biomass prior to use for metal removal from contaminated media can significantly improve their uptake abilities. It was reported that the pre-treated biomass of the marine algae *Padina* sp. has the ability to adsorb Cd at the rate of 90% in 35 min [18]. Moreover, optimization conditions (i.e. pH, pollutant initial concentration, and adsorbent dosage) maximizing phosphorus biosorption (89.2% equivalent to 3.6 mg/g) by the modified freshwater plant *Lemna minor* [19] while acid modified pumice showed higher adsorption capacity than natural pumice in the same conditions [20]. It was suggested that modified *L. minor* and acid modified pumice have high potential for biosorption and can be successfully used as low-cost and effective absorbent for decontaminating aqueous solution. In contrast, living algal and cyanobacterial cells were more efficient in removing nickel at a concentration < 20 ppm from a chemically complex wastewater effluent generated by electroplating operations [21]. However, although dead algae have been utilized successfully in heavy metal adsorption experiments, living algae may be more advantageous due to their metabolic uptake and continuous growth [22].

The ability of microbial masses for heavy metals removal can be enhanced by fixation into (immobilized) or onto (biofilm) selected supporting materials. A packed bed column was shown earlier as the most effective practical application for heavy metal sorption where it had high sorbent capacity and results in a better quality of the effluent [22]. However, the utilization of fixed biomaterials to adsorb metal ions in column mode has not been very often reported. Among the very few available studies, a mini-column of 0.5 cm (diameter) × 1.6 cm (length) packed with *Sargassum* sp. was used, which exhibited Cu and Zn uptake capacities of 11.9 and 21.0 mg g^{-1}, respectively [23]. Other examples include removal capacities of 4.06, 3.76, 0.36, and 1.36 mg g^{-1} of Pb, Cd, Ni, and Zn respectively using a plastic column (1.27 × 40 cm) packed with immobilized *Mucor rouxii* biomass (4.5 g) [24], Cu biosorption capacities (49.58 and 52.76 mg g^{-1}) at different initial copper feed concentrations using a glass column of 1 cm diameter packed with 1g of *Padina* sp. biomass [25] and Cu-biosorption capacity (38 mg g^{-1}) during seven regeneration cycles using a column (2.5 × 50 cm) packed with 38 g of dry *Sargassum filipendula* biomass [26]. Immobilization of microalgae remarkably enhanced their ability for decontamination of polluted effluent as well as offering simple harvesting method of immobilized microalgae through filtration [8]. For example, cultivation of immobilized *Chlorella vulgaris* in the severely polluted palm oil mill effluent (POME), not only protect this microalga from the included toxicity but also enhanced bioremoval of Fe(II) and Mn(II), chemical oxygen demand (95%–99.9%), biochemical oxygen demand (97%–99.9%), total nitrogen (78%–98%), and total phosphate (79%–98%) [27]. This fact was confirmed when comparing biosorption of Fe (II), Mn (II), and Zn (II) ions, from aqueous solution with free (non-immobilised) and Ca-alginate immobilised *C. vulgaris* biomass where immobilized cells showed the maximum biosorption of the tested metals but both cell types confirmed to be feasible, spontaneous and exothermic in nature under the tested conditions [28]. Biosorption capacity of algal cells was strongly dependent on the operational condition such as pH, initial metal ions concentration, dosages, contact time, and temperature. Metal biosorption by this alga was attributed to the presence of the metal binding functional groups C–N, –OH, COO–, –CH, C=C, C=S, and –C– as well as the porous morphology which greatly helps in the biosorption of heavy metals [29].

In the present study, biosorption and removal of four metals (Fe, Zn, Pb, and Cu) from contaminated plastic and electrical industrial effluents by individual and mixed cultures of *Anabaena variabilis* and *Tolypthrix ceytonica* immobilized in sodium alginate were investigated in a continuous mode.

2. Materials and methods

Based on their efficiency for metals removal as free living individuals (data not shown), two living species of the tested cyanobacteria namely *Anabaena variabilis* and *Tolypthrix* *ceytonica* were selected for continuous heavy metals bioremediation of industrial effluents using free and sodium alginate immobilized cells either as individual or mixed cultures.

2.1. Cyanobacteria

Anabaena variabilis (unialgal) and *Tolypthrix ceytonica* (axenic, bacteria-free) strains were kindly provided by Prof. Yahia Azab, Algal Culture Collection of El-Mansoura University. Cultures were kept under optimum incubation conditions of temperature and light throughout the study duration.

2.2. Purification of cyanobacterial strains

Since bacterial contamination that lives in symbiotic relations (i.e. benefit from each other) in the miscellaneous sheath of cyanobacteria can affect the results leading to artifacts, it is very important to purify the selected cultures. Agar phototaqtic response method was performed for *Anabaena variabilis* (unialgal) strain to obtain axenic culture [30].

2.3. Immobilization of cyanobacteria

Biomass of *Anabaena variabilis* and *Tolypthrix ceytonica* (17.2 and 14.8 mg/l wet weight respectively) (Fig. 1) and their mixed culture from early-stationary cultures (15 d) were harvested by centrifugation at 3,000 rpm for 20 min. Immobilization of cyanobacteria carried out as explained in Fig. 2. In that procedure, sodium alginate (3 g) was added to 100 ml MBL liquid medium and autoclaved at 121°C for 20 min. After cooling to room temperature, cyanobacterial suspension either single cell or mixed culture was added at ratio of 1:1 by volume and shacked well for 5 min. Cyanobacteria-alginate suspension was then passed through 50 ml burette into cylinder containing 200 ml 0.03 M $CaCl_3$ placed in a cooling water bath to keep temperature in the range of 1°C–3°C to allow the formation of cyanobacterial alginate beads. Transfer the beads to a beaker containing cold 0.03 M $CaCl_3$ solution and left to stand for 30–60 min after which they were washed with MBL medium at least 3 times and transferred to MBL containing beaker. Immobilized beads were kept in dark at 4°C.

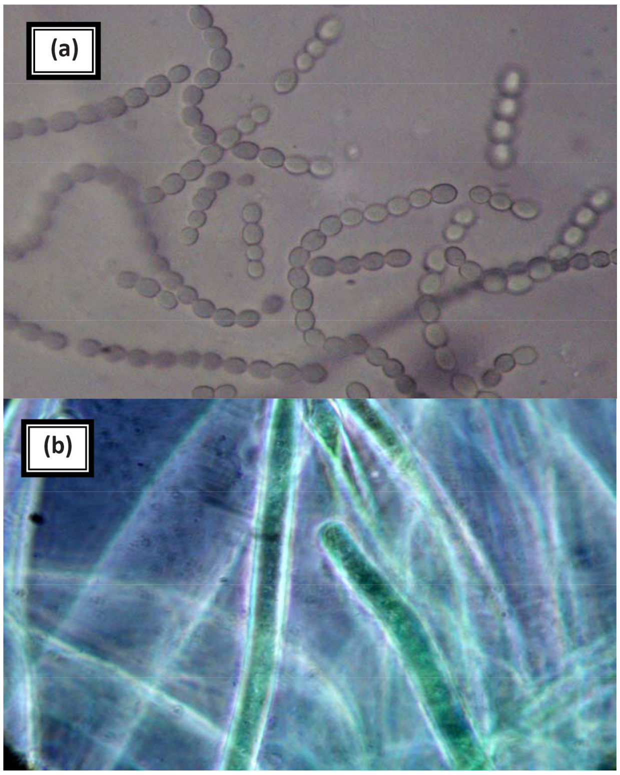

Fig. 1. Selected cyanobacteria used for metals bio-removal (a) *Anabaena variabilis* and (b) *Tolypthrix ceytonica.*

After immobilization, individual or mixed cyanobacterial cultures were packed into three (one culture each) cylindrical glass columns (2 × 40 cm) that sealed at the bottom by a porous metal net (d < 1 mm) and supplied with a flow controller (tap) at the outlet (Fig. 3). A fourth column was set as a control and packed with beads only without cyanobacteria.

2.4. Source and collection of the contaminated industrial effluents

After a through survey of metal contents of various industrial effluents around Alexandria, selection was made on Egyptian Plastic and Electrical Industries Company (Varta), Alexandria due to the significant metal content in its discharges. Samples were collected directly from the final drainage effluent in overnight pre-acid-washed polyethylene containers (10 % HCl) during the working hours of the factory at three different dates. Heavy metals in the raw effluents were determined and averaged. Levels of Cu, Fe, Zn, and Pb (the selected metals) in the raw effluent recorded 0.15, 13.88, 5.1, and 4.5 mg/l respectively at nearly neutral pH.

2.5. Batch treatment using free cyanobacteria

Before the application of the continuous treatment using immobilized cyanobacteria, *Anabaena variabilis* and *Tolypthrix*

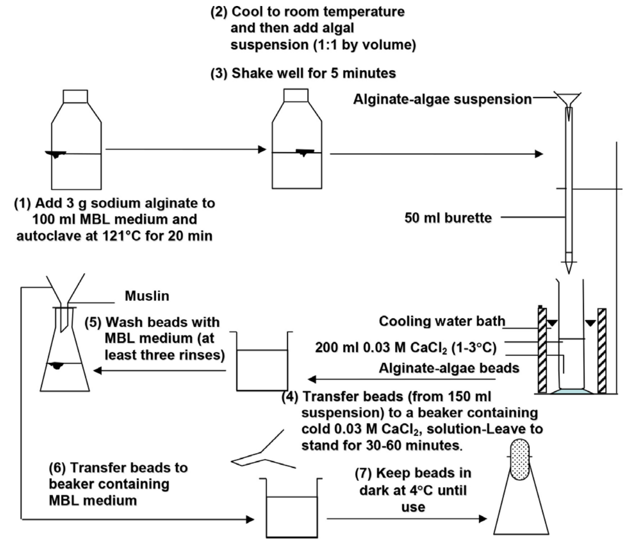

Fig. 2. Immobilization of the selected cyanobacteria using sodium alginate.

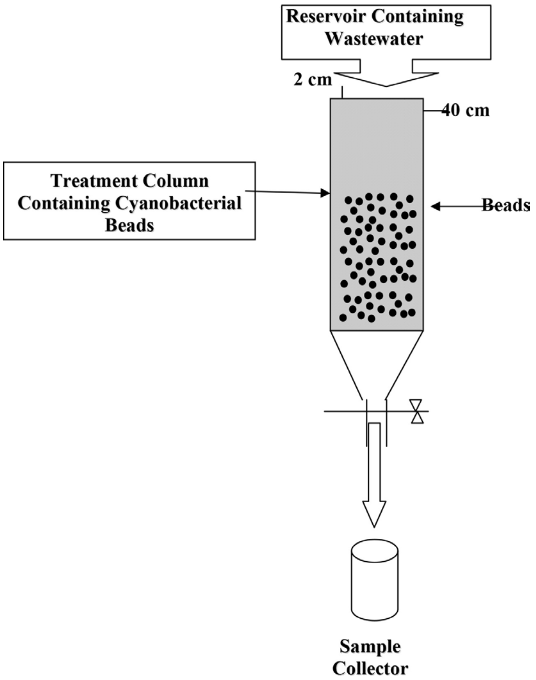

Fig. 3. Diagrammatic sketch of the proposed treatment system.

ceytonica as well as their mixture were examined as free living cultures in batch treatment for the contaminated industrial effluent. Ten flasks (1 L) were prepared with 200 ml of artificial culture medium (MBL) each (optimal for cyanobacteria growth) and sterilized by autoclaving for 20 min at 121°C. *Anabaena variabilis* and *Tolypthrix ceytonica* as well as their mixture were inoculated individually under sterile conditions (3 replicas each) with 1 g fresh wet biomass at the end of the logarithmic phase of each of the tested cyanobacteria while the tenth represents a control (cyanobacteria-free) culture. Then all cultures were incubated for 24 h at 25°C ± 2ºC and 150 rpm shacking speed under continuous illumination using cool fluorescent light at 50 µmol photon m^{-2} s^{-1} irradiance. Raw industrial effluent (800 ml) was added to each flask to a final volume of 1 L. The experiment was carried out for six consecutive hours where samples were collected for heavy metal analysis on hourly interval.

2.6. Operation conditions of the continuous treatment using immobilized cyanobacteria

Raw samples were treated using the proposed system at two different flow rates (50 and 100 ml/h). These rates were selected based on a pilot primary experiment, which indicated that among other flow rates tested, 50 and 100 were optimal for such kind of wastewater. At each flow rate, samples were collected from both the cyanobacteria and cyanobacteria-free (control) columns on hourly interval for six consecutive hours.

2.7. Heavy metals determination

After treatment, treated samples as well as raw water were characterized for the selected dissolved heavy metals using atomic absorption spectrophotometer (AAS) according to the standard method described in the "Standard Methods for Examination of Water and Wastewater" [31]. Water samples were filtered through membrane filter and subjected to analysis on individual basis using specific lamp with specific wavelength. The efficiencies of the heavy metals removal using the proposed system were calculated.

3. Results

Analyzing the polluted industrial wastewater used in this work revealed the presence of four heavy metals namely copper, iron, zinc, and lead with high concentrations. Their levels reported 0.15, 13.88, 5.1, and 4.5 mg/l for Cu^{2+}, Fe^{3+}, Zn^{2+}, and Pb^{2+} respectively. Based on the batch experiments performed to study the ability of six selected cyanobacterial species for removing the five selected metals (data are not shown), *Anabaena variabilis* followed by *Tolypthrix ceytonica* represent the most promising species for remediation of metals-contaminated effluents. Therefore *Anabaena variabilis* and *Tolypthrix ceytonica* were chosen to study their capacity to remove Cu^{2+}, Fe^{3+}, Zn^{2+}, and Pb^{2+} ions from the contaminated industrial wastewater either as individual or mixed immobilized cultures.

3.1. Application of free cyanobacteria for heavy metals bioremediation

Results of the residual concentrations and removal efficiencies of the four metals contaminating industrial wastewater by the individual or mixed free cultures of *Anabaena variabilis* and *Tolypthrix ceytonica* (Table 1) revealed that RE% is a function of the type of heavy metal, exposure time, and microbial species. RE(s)% of all the investigated metals are proportionally increased with exposure time regardless metal type, microbial species or metal concentration. Consequently, the highest removal percentage was recorded at the end of the experiment (6 h).

The maximum RE(s)% ranges achieved by the tested individual cultures after six exposure hours were 92.35–98.23 for Zn^{2+} followed by Fe^{3+} (79.09–97.22), Pb^{2+} (37.11–97.95) then finally Cu^{2+} (85.33–89.33) with the lowest maximum RE% confirming the importance of the metal type in the removal process and indicating the relative toxicity for these metals.

The ranges of RE(s)% achieved for Cu^{2+} are 20.67–89.33; 51.33–85.33 and 34.67–88.00 by *A. variabilis; T. ceytonica* and the mixed culture of *A. variabilis and T. ceytonica* respectively. These ranges confirmed the high ability of *A. variabilis* over the two other cultures for Cu^{2+}removal followed by the mixed culture and then *T. ceytonica*. Fe^{3+} RE(s)% ranges recorded 68.59–93.44; 70.02–97.22, and 66.89–79.09 for the three cultures respectively with *T. ceytonica* achieving the highest ranges followed by *A. variabilis* and finally the mixed culture. Zn^{2+} exhibited RE(s)% of 86.47–98.23; 79.21–93.33, and 85.88–92.35 by the three cultures respectively with *A. variabilis* recorded higher Zn^{2+} removal over other cultures followed by the mixed culture especially at exposures up to 5 h then *T. ceytonica.* Pb^{2+} showed the same behavior like Fe^{3+} with *T. ceytonica* achieving the highest ranges (4.89–97.95) followed by *A. variabilis* (2.67–86.67) and then the mixed culture (2.22–37.11) with the lowest removal range. Variations in the removal of the same metals by different organisms even from the same group are

Table 1
Residual concentrations (RC) and removal efficiency (RE%) of some heavy metals contaminating industrial wastewater using individual and mixed free living cultures of *Anabaena variabilis* and *Tolypthrix ceytonica* at different exposure times

Heavy metal	Time (h)	*A. variabilis*		*T. ceytonica*		*A. variabilis* and *T. ceytonica*		*p*
		RC^{a}	RE%	RC	RE%	RC^{a}	RE%	
Cu	0	0.150±0.005^{a}	–	– ± –	–	– ± –	–	
	1	0.119±0.004	20.67*	0.073±0.002	51.33*	0.098±0.003	34.67*	0.032
	2	0.107±0.004	28.67	0.056±0.002	62.67	0.053±0.002	64.67	0.011
	3	0.039±0.001	74.00	0.050±0.002	66.67	0.050±0.001	66.67	>0.05
	4	0.036±0.001	76.00	0.045±0.001	70.00	0.045±0.001	70.00	>0.05
	5	0.018±0.001	88.00	0.038±0.001	74.67	0.053±0.002	71.33	>0.05
	6	0.016±0.001	89.33**	0.022±0.001	85.33**	0.018±0.001	88.00**	>0.05
r, p		0.98, 0.0021^{b}		0.74, 0.012^{b}		0.71, 0.023^{b}		
Fe	0	13.88±0.458	–	– ± –	–	– ± –	–	
	1	4.359±0.144	68.59*	4.161±0.137	70.02*	4.596±0.152	66.89*	>0.05
	2	4.327±0.143	68.82	4.011±0.132	71.10	4.405±0.145	68.26	>0.05
	3	3.770±0.124	72.84	3.203±0.106	76.92	4.271±0.140	69.23	>0.05
	4	3.061±0.101	77.95	2.868±0.095	79.34	4.006±0.132	71.14	>0.05
	5	2.196±0.072	84.18	1.789±0.059	87.11	3.115±0.102	77.56	>0.05
	6	0.911±0.03	93.44**	0.385±0.017	97.22**	2.902±0.095	79.09**	0.013
r, p		0.92, 0.001^{b}		0.95, 0.001^{b}		0.75, 0.0025^{b}		
Zn	0	5.10±0.168	–	– ± –	–	– ± –	–	
	1	0.69±0.023	86.47*	1.060±0.034	79.21*	0.720±0.023	85.88*	>0.05
	2	0.55±0.018	89.21	0.650±0.021	87.25	0.600±0.019	88.23	>0.05
	3	0.26±0.009	94.90	0.550±0.018	89.21	0.480±0.015	90.59	>0.05
	4	0.22±0.007	95.69	0.480±0.015	90.59	0.440±0.014	91.37	>0.05
	5	0.11±0.004	97.84	0.450±0.014	91.18	0.420±0.013	91.77	>0.05
	6	0.09±0.003	98.23**	0.340±0.011	93.33**	0.390±0.012	92.35**	>0.05
r, p		0.98, 0.001^{b}		0.88, 0.0031^{b}		0.66, 0.039^{b}		
Pb	0	4.5	–	– ± –	–	– ± –	–	
	1	4.50±0.149	2.67*	4.280±0.141	4.89*	4.400±0.145	2.22*	0.041
	2	4.38±0.145	20.89	3.230±0.106	28.22	4.320±0.142	4.00	0.021
	3	3.56±0.117	55.33	2.700±0.089	40.00	4.100±0.135	8.88	0.001
	4	2.01±0.066	62.00	2.220±0.073	50.67	4.040±0.133	10.22	0.001
	5	1.71±0.056	64.89	1.050±0.034	76.67	2.930±0.096	34.89	0.012
	6	1.58±0.052	86.67**	0.092±0.003	97.95**	2.830±0.093	37.11**	0.001
r, p		0.92, 0.001^{b}		0.98, 0.001^{b}		0.88, 0.0102^{b}		

^{a}Average value ± SE.
^{b}Significance of variance at confidence level of 0.05 and 0.01 (2-tailed).
*The lowest recorded RE%.
** The highest recorded RE%.

attributed mainly to the selectivity and different affinities of such organisms to different metals based on their metabolic capabilities and resistance and/or tolerance to such metals. This fact is clearly shown in the present study.

Therefore, results concluded that free individual and mixed cultures of the two selected species could remove the tested metals with very high RE(s)% in the following order $Zn^{2+} > Fe^{3+} > Pb^{2+} > Cu^{2+}$ and at a very high rate. Also high selectivity was observed among the tested cultures. This was shown by the highest removal of Zn^{2+} and Cu^{2+}achieved by *A. variabilis* while the highest removal of Fe^{3+} and Pb^{2+} was achieved by *T. ceytonica.* The mixed culture almost showed lower RE% for the tested metals confirming that individual cultures of the selected species are more active in metals removal.

3.2. Application of immobilized cyanobacteria for heavy metals bioremediation

Tables 2–5 and Figs. 4–7 represent residual concentrations and removal efficiencies of the four metals contaminating industrial wastewater by the individual or mixed immobilized cultures of *Anabaena variabilis* and *Tolypthrix ceytonica.* Generally immobilized cells of all cultures achieved higher RE(s)% for all the tested metals at all the exposure times and flow rates compared with their corresponding free cells cultures.

Again and as shown with the free living cultures, RE% was a function of the type of heavy metal, microbial species and exposure time achieving their highest removal percentage at the end of the experiment (6 h) regardless metal type, microbial species or metal concentration.

For Fe^{3+}, very regular behavior was exhibited by all the tested cultures. They all showed their maximum RE(s)% after 6 h (Fig. 4) at the slowest flow rate of 50 ml/h {94.45 (*A. variabilis*); 67.44 (*A. variabilis and T. ceytonica)* and 59.58

Table 2
Residual concentrations (RC) and removal efficiency (RE%) of Iron (Fe^{3+}) contaminating industrial wastewater using immobilized individual and mixed cultures of *Anabaena variabilis* and *Tolypthrix ceytonica* at different exposure times and flow rates

Cyanobacteria	Time (h)	Flow rate 50 ml/h		Flow rate 100 ml/h		
		RC[a]	RE%	RC[a]	RE%	*p*
A. variabilis	0	13.88±0.499	–	–	–	–
	1	9.72±0.341	29.97*	9.54±0.339	31.26*	>0.05
	2	8.02±0.272	42.22	8.99±0.305	35.22	>0.05
	3	7.84±0.274	43.52	8.79±0.307	36.66	>0.05
	4	5.72±0.201	58.79	7.93±0.277	42.85	0.001
	5	3.66±0.128	73.63	6.55±0.229	52.80	0.0023
	6	0.77±0.026	94.45**	5.23±0.183	62.31**	>0.05
r, p		0.69, 0.01[b]		0.68, 0.02[b]		
T. ceytonica	0	13.88±0.499	–	–	–	
	1	8.85±0.309	36.24*	9.12±0.319	34.29*	>0.05
	2	8.82±0.299	36.46	9.11±0.309	34.36	>0.05
	3	8.61±0.301	37.97	8.93±0.312	35.66	>0.05
	4	8.40±0.294	39.48	8.53±0.298	38.53	>0.05
	5	7.30±0.255	47.41	7.94±0.277	42.77	>0.05
	6	13.88±0.499	59.58**	7.33±0.216	47.18**	>0.05
r, p		0.22, 0.39[b]		0.41, 0.103[b]		
A. variabilis and *T. ceytonica*	0	13.88±0.499	–	–	–	–
	1	11.81±0.413	14.91*	13.08±0.457	5.75*	0.0025
	2	7.66±0.261	44.81	12.55±0.426	9.55	0.0001
	3	7.33±0.256	47.19	10.89±0.381	21.53	0.002
	4	6.71±0.234	51.66	9.58±0.335	30.95	0.001
	5	5.67±0.198	59.15	8.56±0.299	38.29	0.0032
	6	4.52±0.158	67.44**	7.05±0.246	49.18**	0.004
r, p		0.71, 0.0102[b]		0.62, 0.025[b]		

[a] Average value ± SE.
[b] Significance of variance at confidence level of 0.05 and 0.01 (2-tailed).
*The lowest recorded RE%.
**The highest recorded RE%.

Table 3
Residual concentrations (RC) and removal efficiency (RE%) of Zinc (Zn^{2+}) contaminating industrial wastewater using immobilized individual and mixed cultures of *Anabaena variabilis* and *Tolypthrix ceytonica* at different exposure times and flow rates

Cyanobacteria	Time (h)	Flow rate 50 ml/h		Flow rate 100 ml/h		
		RC[a]	RE%	RC[a]	RE%	*p*
A. variabilis	0	5.100±0.184	–	–	–	
	1	0.211±0.007	95.86*	0.195±0.006	96.17*	>0.05
	2	0.125±0.004	97.55	0.085±0.002	98.33	>0.05
	3	0.121±0.004	97.63	0.071±0.002	98.61	>0.05
	4	0.107±0.003	97.90	0.058±0.001	98.86	>0.05
	5	0.106±0.003	97.92	0.057±0.002	98.88	>0.05
	6	0.087±0.002	98.29**	0.052±0.001	98.98**	>0.05
r, p		0.71, 0.003[b]		0.87, 0.001[b]		
T. ceytonica	0	5.100±0.183	–	–	–	
	1	0.187±0.006	96.33*	0.124±0.004	97.57*	>0.05
	2	0.163±0.005	96.80	0.112±0.004	97.80	>0.05
	3	0.150±0.005	97.06	0.109±0.003	97.86	>0.05
	4	0.146±0.004	97.14	0.092±0.003	98.19	>0.05
	5	0.141±0.004	97.23	0.081±0.002	98.41	>0.05
	6	0.112±0.003	97.80**	0.071±0.002	98.61**	>0.05
r, p		0.15, 0.58[b]		0.22, 0.41[b]		
A. variabilis and *T. ceytonica*	0	5.100±0.183	–	–	–	
	1	0.137±0.004	97.31*	0.142±0.005	97.21*	>0.05
	2	0.126±0.004	97.53	0.111±0.003	97.82	>0.05
	3	0.125±0.004	97.55	0.081±0.003	98.41	>0.05
	4	0.117±0.003	97.70	0.078±0.002	98.47	>0.05
	5	0.116±0.003	97.72	0.072±0.002	98.59	>0.05
	6	0.088±0.002	98.27**	0.070±0.002	98.63**	>0.05
r, p		0.61, 0.021[b]		0.58, 0.041[b]		

[a] Average value ± SE.
[b] Significance of variance at confidence level of 0.05 and 0.01 (2-tailed).
*The lowest recorded RE%.
**The highest recorded RE%.

(*T. ceytonica*)} which declined significantly by increasing the flow rate up to the highest investigated rate of 100 ml/h {62.31 (*A. variabilis*); 49.18 (*A. variabilis and T. ceytonica)* and 47.18 (*T. ceytonica*)}.

Concerning Zn^{2+}, insignificant differences were recorded in the RE(s)% at both flow rates 50 and 100 ml/h by all cultures at all the exposure times (Fig. 5). The maximum achieved RE(s)% for Zn^{2+} after 6 h at 50 ml/h flow rate recorded 98.29 (*A. variabilis*); 98.27 (*A. variabilis and, T. ceytonica),* and 97.80 (*T. ceytonica*). These levels were slightly increased at 100 ml/h to record maximum RE(s)% of 98.98 (*A. variabilis*); 98.63 (*A. variabilis and T. ceytonica),* and 98.61 (*T. ceytonica*).

Lead (Pb^{2+}) showed similar behavior like Zn^{2+} where higher RE(s)% were recorded at the highest flow rate of 100 ml/h compared with those obtained at 50 ml/h (Fig. 6). However, unlike Zn^{2+}, Pb^{2+} exhibited highly significant differences in the RE(s)% between the two flows at all exposures by all cultures. The maximum achieved RE(s)% for Pb^{2+} after 6 h at 50 ml/h recorded 87.77 (*A. variabilis* and *T. ceytonica*); 66.00 (*A. variabilis*), and 59.33 (*T. ceytonica*). These levels were significantly increased at 100 ml/h to record maximum RE(s)% of 94.22 (*A. variabilis and T. ceytonica*); 88.00 (*T. ceytonica),* and 82.89 (*A. variabilis*).

Copper (Cu^{2+}) uptake by the investigated cultures was more or less similar to that of iron (Fe^{3+}) where they all showed their maximum RE(s)% after six exposure hours at 50 ml/h which declined significantly by increasing the flow rate up to 100 ml/h (Fig. 7). The maximum achieved RE(s)% for Cu^{2+} at 50 ml/h are 93.33 (*A. variabilis and T. ceytonica*); 92.00 (*A. variabilis),* and 89.33 (*T. ceytonica*). These levels were decreased at 100 ml/h to record maximum RE(s)% of 91.33 (*A. variabilis and T. ceytonica*); 89.33 (*A. variabilis),* and 84.00 (*T. ceytonica*).

Metals contaminated the industrial effluent as a mixture were removed by the immobilized cyanobacteria in the following order Zn^{2+} > Cu^{2+} > Fe^{3+} > Pb^{2+} at 50 ml/h and Zn^{2+} > Pb^{2+} > Cu^{2+} > Fe^{3+} at 100 ml/h. Ranges of RE(s)% achieved for

Table 4
Residual concentrations (RC) and Removal efficiency (RE%) of Lead (Pb^{2+}) contaminating industrial wastewater using immobilized individual and mixed cultures of *Anabaena variabilis* and *Tolypthrix ceytonica* at different exposure times and flow rates

Cyanobacteria	Time (h)	Flow rate 50 ml/h		Flow rate 100 ml/h		
		RC[a]	RE%	RC[a]	RE%	*p*
A. variabilis	0	4.5	–	–	–	
	1	4.50±0.162	50.89*	0.98±0.034	78.22*	0.025
	2	2.21±0.077	55.55	0.96±0.032	78.66	0.031
	3	2.00±0.068	61.33	0.91±0.031	79.78	>0.05
	4	1.74±0.060	62.44	0.87±0.030	80.67	>0.05
	5	1.69±0.059	65.55	0.82±0.028	81.78	>0.05
	6	1.55±0.054	66.00**	0.77±0.026	82.89**	>0.05
r, p		0.62, 0.012[b]		0.74, 0.032[b]		
T. ceytonica	0	4.50±0.162	–	–	–	
	1	2.34±0.081	48.00*	2.15±0.075	52.22*	>0.05
	2	2.29±0.078	49.11	1.18±0.040	73.78	0.021
	3	2.20±0.077	51.11	0.92±0.032	79.55	0.024
	4	2.02±0.070	55.11	0.77±0.026	82.89	0.001
	5	1.97±0.068	6.22	0.72±0.025	84.00	0.0021
	6	1.83±0.064	59.33**	0.54±0.018	88.00**	0.0032
r, p		0.59, 0.049[b]		0.61, 0.041[b]		
A. variabilis and *T. ceytonica*	0	4.50±0.162	–	–	–	
	1	1.20±0.042	73.33*	1.38±0.048	69.33*	>0.05
	2	1.11±0.037	75.33	0.70±0.023	84.44	>0.05
	3	0.90±0.031	80.00	0.63±0.022	88.00	>0.05
	4	0.81±0.028	82.00	0.42±0.014	90.67	>0.05
	5	0.80±0.028	82.22	0.35±0.012	92.22	>0.05
	6	0.55±0.019	87.77**	0.26±0.009	94.22**	>0.05
r, p		0.79, 0.002[b]		0.71, 0.01[b]		

[a] Average value ± SE.
[b] Significance of variance at confidence level of 0.05 and 0.01 (2-tailed).
*The lowest recorded RE%.
** The highest recorded RE%

these metals by the present immobilized cultures exhibited superior ability and an efficient tool for decontaminating highly toxic metals such as Cu^{2+}, Cd^{2+}, and Pb^{2+}.

Concerning the efficiency order of the selected cyanobacterial cultures, immobilized *A. variabilis* came on the top list for Fe^{3+} and Zn^{2+} removal at the two flow rates followed by the mixed immobilized culture of *A. variabilis and T. ceytonica* and finally *T. ceytonica*. While for Cu^{2+} and Pb^{2+} (the most toxic) were highly removed by the mixed immobilized culture of *A. variabilis and T. ceytonica* followed mostly by *A. variabilis* and finally *T. ceytonica*. This confirmed the selective uptake by the different cultures as well as the importance of immobilization for improving the removal efficiencies of the contaminated metals.

4. Discussion

Heavy metals polluting wastewater can be treated using a variety of technologies, including chemical, physical, and biological. A promising method using living aquatic plants to sorb metals from water as an alternative process to physiological and chemical has been called biosorption, bioremoval, bio-separation, and sometimes phytoremediation [32]. Wastewater arising from electroplating, electronics, and metal cleaning industries often contains excessive amount of heavy metals and causes serious water pollution problems. Microalgae have been found to be very effective in removing heavy metals from wastewater because of their large surface area and high binding affinity. Also, microalgae have

Table 5
Residual concentrations (RC) and Removal efficiency (RE%) of Copper (Cu^{2+}) contaminating industrial wastewater using immobilized individual and mixed cultures of *Anabaena variabilis* and *Tolypthrix ceytonica* at different exposure times and flow rates

Cyanobacteria	Time (h)	Flow rate 50 ml/h		Flow rate 100 ml/h		
		RC[a]	RE%	RC[a]	RE%	*p*
A. variabilis	0	0.150±0.0054	–	–	–	
	1	0.050±0.0017	66.67*	0.033±0.0011	78.00*	>0.05
	2	0.037±0.0012	75.33	0.025±0.0008	83.33	>0.05
	3	0.034±0.0011	77.33	0.023±0.0008	84.67	>0.05
	4	0.017±0.0005	88.67	0.018±0.0006	88.00	>0.05
	5	0.013±0.0004	91.33	0.017±0.0005	88.67	>0.05
	6	0.012±0.0004	92.00**	0.016±0.0005	89.33**	>0.05
r, p		0.76, 0.0041[b]		0.75, 0.0032[b]		
T. ceytonica	0	0.150±0.0054	–	–	–	
	1	0.108±0.0037	28.00*	0.138±0.0048	8.00*	0.0023
	2	0.055±0.0018	63.33	0.120±0.0040	20.00	0.0001
	3	0.038±0.0013	74.67	0.088±0.0030	41.33	0.0001
	4	0.029±0.0010	80.67	0.061±0.0021	60.00	0.032
	5	0.020±0.0007	86.67	0.043±0.0015	71.44	>0.05
	6	0.016±0.0005	89.33**	0.024±0.0008	84.00**	>0.05
r, p		0.75, 0.024[b]		0.77, 0.012[b]		
A. variabilis and *T. ceytonica*	0	0.150±0.0054	–	–	–	
	1	0.044±0.0015	70.67*	0.079±0.0027	47.33*	0.021
	2	0.020±0.0007	86.67	0.069±0.0023	54.00	0.001
	3	0.018±0.0006	88.00	0.038±0.0013	74.66	>0.05
	4	0.017±0.0005	88.67	0.025±0.0008	83.33	>0.05
	5	0.016±0.0005	89.33	0.018±0.0006	88.00	>0.05
	6	0.010±0.00036	93.33**	0.013±0.0004	91.33**	>0.05
r, p		0.71, 0.032[b]		0.65, 0.044[b]		

[a] Average value ± SE.
[b] Significance of variance at confidence level of 0.05 and 0.01 (2-tailed).
*The lowest recorded RE%.
**The highest recorded RE%.

a negative surface charge and therefore they possess a high affinity for heavy metal ions, which make them especially effective in wastewater detoxification [33].

Treatment of the raw effluent of Varta Company contaminated with copper, iron, zinc and lead using the two highly resistant cyanobacterial species *Anabaena variabilis* and *Tolypthrix ceytonica* free or immobilized, individual or mixed cultures indicated a very successful tool. They showed an increasing RE(s)% for all the contaminating metals at a very high rate (6 h) which can be reduced by using serial units in sequence. Free *Anabaena variabilis* culture achieved 89.33%, 93.44%, 98.23%, and 86.67% removal of copper, iron, zinc, and lead from the wastewater respectively compared with 85.33%, 97.22%, 93.33%, and 97.95% by *Tolypthrix ceytonica* and 88.00%, 79.09%, 92.35%, and 37.11% respectively by the mixed culture. Results indicated that RE(s)% depends on the type of heavy metal, incubation period and microbial species. This was confirmed by Tien [34] who recorded that competition of metal ions on algal surface binding sites differed with algal species and metal ions. Metal sorption efficiency depends on the type of biosorbent, the physiological state of the cells, availability and concentration of heavy metal and chemical composition of wastewater [35]. The high and significantly different sorption activities for copper, cadmium, and lead by the four algae in this study suggested the suitability and good selectivity for treatment of different kinds of industrial effluents. Results revealed also that individual cultures are almost better than the mixed cultures in heavy metal removal which may be attributed to the competition for nutrients among cyanobacterial species in the mixed cultures. However, for application of microbial biomass to sorb metal ions during the continuous industrial process, it

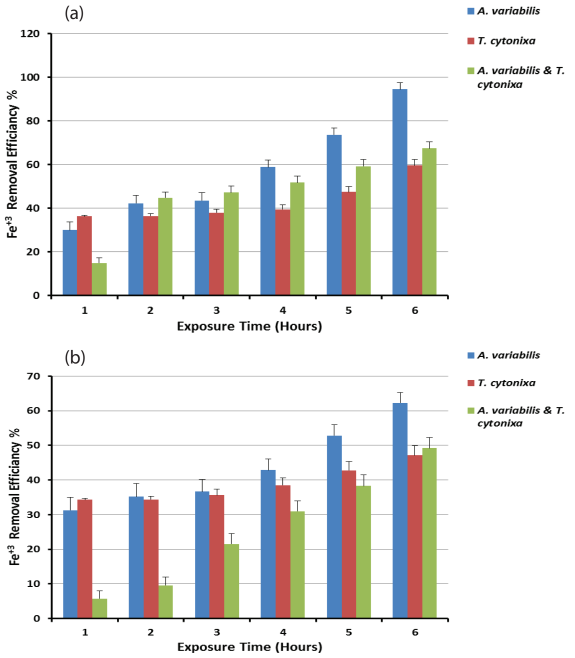

Fig. 4. Removal efficiency (RE%) of iron (Fe^{3+}) contaminating industrial wastewater using individual and mixed cultures of immobilized *Anabaena variabilis* and *Tolypthrix ceytonica* at different exposure times and flow rates. (a) Flow rate 50 ml/h and (b) Flow rate 100 ml/h.

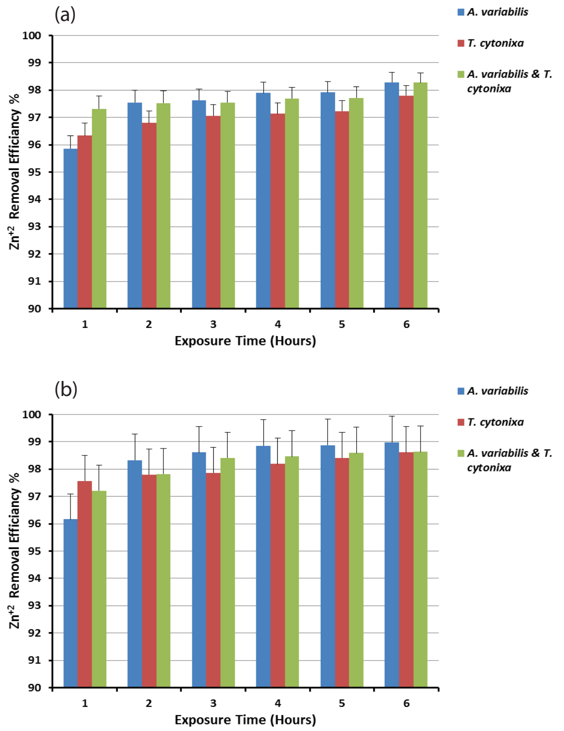

Fig. 5. Removal efficiency (RE%) of Zinc (Zn^{2+}) contaminating industrial wastewater using individual and mixed cultures of immobilized *Anabaena variabilis* and *Tolypthrix ceytonica* at different exposure times and flow rates. (a) Flow rate 50 ml/h and (b) Flow rate 100 ml/h.

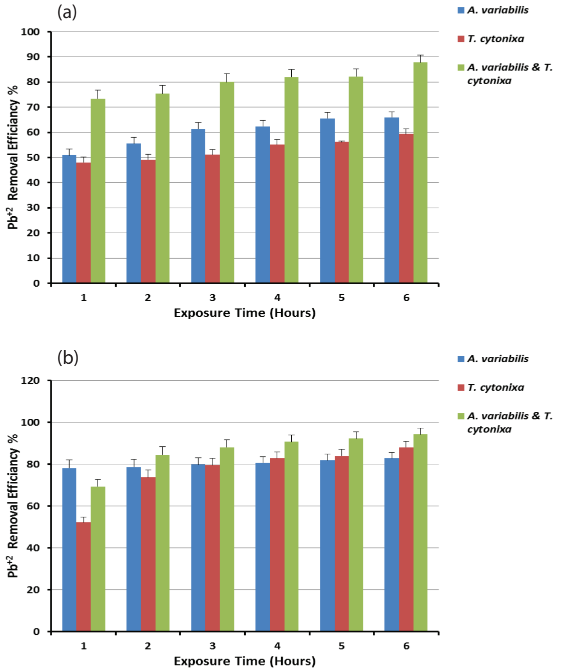

Fig. 6. Removal efficiency (RE%) of lead (Pb^{2+}) contaminating industrial wastewater using individual and mixed cultures of immobilized *Anabaena variabilis* and *Tolypthrix ceytonica* at different exposure times and flow rates. (a) Flow rate 50 ml/h and (b) Flow rate 100 ml/h.

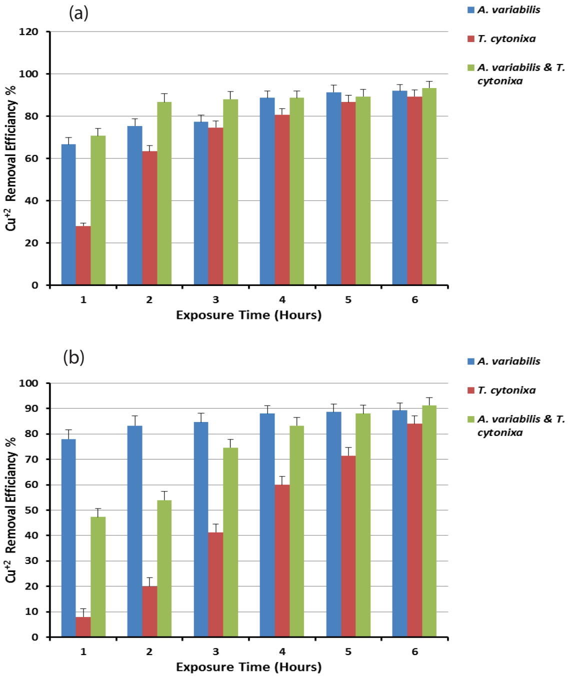

Fig. 7. Removal efficiency (RE%) of copper (Cu^{2+}) contaminating industrial wastewater using individual and mixed cultures of immobilized *Anabaena variabilis* and *Tolypthrix ceytonica* at different exposure times and flow rates. (a) Flow rate 50 ml/h and (b) Flow rate 100 ml/h.

is important to utilize an appropriate immobilization technique. Immobilized biomass offers many advantages including better reusability, high biomass loading and minimal clogging in continuous flow systems [36,37]. Immobilization appears to be one of the best techniques to physically separate micro-algal cells from their culture medium for the purpose of algal tertiary wastewater treatment. Moreover, by using immobilization on screens, removal of nutrients from wastewater was higher than with conventional biological tertiary wastewater treatments [27–29,38].

Immobilization of microorganisms is a current topic in biotechnology. Among the different immobilization techniques, calcium alginate matrix is one of the most used [8,27–29,39]. Calcium alginate is nontoxic and permits different types of microorganisms to grow inside. The transparency of small calcium-alginate beads is enough to permit the growth of immobilized microalgae [40]. Additionally, it is an easy, cheap and feasible technique to be used in research laboratories [8,27–29,41]. Removal efficiency of copper, iron, zinc, and lead from the contaminated effluent by the immobilized cultures of *A. variabilis* and *T. ceytonica* either individual or as a mixed culture in the continuous treatment recorded higher RE(s)% compared with the treatment with the free cells and differed according to the flow rate used (50 and 100 ml/h) as well as the type of heavy metal, incubation period, and the tested species. This may be directly attributed to increasing protection and resistance of immobilized cells against wastewater toxicity compared with their free living cells. In contrast, biosorption of metal ions by immobilized *Phormidium laminosum* biomass was rapid during the first hour, and then continued at a slower rate for the following several hours [42]. Time of exposure has also been reported as a factor in uptake levels of heavy metals [43].

Pretreatment (including immobilization) of microbial biomass used as biosorbents considered excellent step for enhancing their biosorption even with highly toxic metals like Cu [26,44,45]. These studies are in consistent with the present results and highlighted the importance of immobilization or microbial fixation in improving remediation capability of the tested organisms.

In case of Fe^{3+}, the flow rate 50 ml/h was more efficient for metals removal compared with 100 ml/h which was previously found for *Ulva* [46]. Results are also in agreement with other workers [47] who reported that competition for nutrients in the mixed cultures affected the total performance of wastewater biotreatment with respect to the heavy metal removal and observed the preferential ability of one organism to remove nutrients more than others.

Removal efficiency of Zn^{2+} using the proposed cyanobacteria immobilized reached nearly 98% under all the conditions tested as also found for immobilized *Microcystis aeruginosa* for metals removal and recovery from contaminated waters [48]. In another study, biosorption rates of up to 96% were recorded for Zn^{2+} within the first 15 min of the reaction at initial concentrations of 110 mg l^{-1} [49]. Lead was highly removed by the mixed culture under the two flow rates with higher removals at the flow rate 100 ml/h compared with flow rate 50 ml/h by the three immobilized cultures. This was totally opposite to Fe^{2+} and Cu^{2+} with the highest removals at flow rate 50 ml/h compared with 100 ml/h. Results also revealed that the bioremoval of lead by the mixed culture was higher than in the individual cultures and the immobilized *T. ceytonica* was more efficient in removing lead from wastewater compared to *A. variabilis*. As early as 1982, it was reported that, the use of sewage effluent contained the heavy metals such as Fe^{3+}, Zn^{2+}, Mn^{2+}, Pb^{2+}, Cd, Ni, Co, and Cr favored the growth of algae and cyanobacteria [50]. Algae have been found to be potential suitable biosorbents because of their cheap availability, relatively high surface area, and high binding affinity [51,52].

5. Conclusion

Metals removal efficiency (RE%) using free or immobilized cyanobacteria is a function of heavy metal type and microbial species and proportionally increased with exposure time. Free individual and mixed cultures of the two selected species (*A. variabilis*; *T. ceytonica*) showed high RE(s)% for the tested metals at a very high rate and selectivity. The maximum achieved RE(s)% after 6 h exposure by batch cultures are; Zn (92.35–98.23), Fe (79.09–97.22), Pb (37.11–97.95) and finally Cu (85.33–89.33) with the lowest maximum RE%. The mixture of the two species almost showed lower RE% for the tested metals compared with their individual cultures. Regarding continuous treatment using the immobilized cyanobacteria, results revealed that RE% of copper, iron and zinc was higher at 50 ml/h compared with that at 100 ml/h while lead also showed high removal efficiency at the flow rate 100 ml/h. *A. variabilis* removed the tested metals in the following order $Zn^{2+} > Fe^{3+} > Cu^{2+} > Pb^{2+}$ at 50 ml/h, while at 100 ml/h the order was $Zn^{2+} > Cu^{2+} > Pb^{2+} > Fe^{3+}$. For *T. ceytonica* the RE% recorded the following order $Zn^{2+} > Cu^{2+} > Fe^{3+} > Pb^{2+}$ and $Zn^{2+} > Pb^{2+} > Cu^{2+} > Fe^{3+}$ at 50 and 100 ml/h respectively. The mixed culture removed these metals in the following orders $Zn^{2+} > Cu^{2+} > Pb^{2+} > Fe^{3+}$and $Zn^{2+} > Pb^{2+} > Cu^{2+} > Fe^{3+}$ at 50 and 100 ml/h respectively.

As a general conclusion, it is clear that the selected cyanobacterial species free or fixed, individual or mixtures characterize by excellent bioremoval abilities toward metals contaminating water or wastewater even at their high concentrations with selective preferences among them. This advantage could be efficiently used for decontaminating wastewater effluents as well as natural aquatic ecosystems. It also provides an economic and excellent tool not only for the protection of the received environments but also to recover and reuse the treated compatible wastewater in any purpose such as irrigation of agricultural non edible crops.

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